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INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. If an injury is not immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. The critical time in shock appears to be the period during which the patient is being resuscitated. Resuscitation is associated with a massive activation of the inflammatory reaction, producing immunosuppression, and rendering the patient vulnerable to sepsis and its sequelae. The *goal* of this research program is to develop new treatments for hemorrhagic shock which can be administered before or during initial resuscitation. These treatments are intended to be applied by front-line responders on the battlefield (and first responders in civilian life) as well as by fixed facilities, such as a Forward Surgical Team or a Combat Support Hospital. These agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers, and must be easy to administer under combat conditions. In previous work, the xanthine oxidase inhibitor allopurinol was found beneficial in a shock model (1). In current work, using an animal model of hemorrhagic shock (2), glutamine, glutamine-alanine dipeptide, and arginine have shown efficacy. (3,4) Studies with crocetin have also shown efficacy (5), but this agent is not available in a form which can be administered to patients. Others have advocated the use of DHEA, but studies in our laboratories have failed to show a useful effect (6). Studies with omega-3 fatty acids have shown promise, but are still at an early stage.

The *method* of the research is to measure the response of the genome to hemorrhagic shock using microarray studies. The effect of these agents in patients will be compared to their effect in the animal model, to assess whether they will be effective in the clinical setting.

BODY

It should be noted that progress towards the four research objectives, while significant, has been slower than would be desired. The microarray studies in the animal models have been slowed by the relocation of the laboratory of the collaborating investigators to new quarters in a newly-built research building. Authorization for human studies was not obtained until June, 2007, and it has proven difficult to find suitable subjects. One of the key individuals, Dr. R. Yang, who carried out the animal studies, returned to China somewhat abruptly and unexpectedly. New personnel are being recruited. On the other hand, the PI (Dr. Van Way) has been named to a research professorship (Sosland Chair of Trauma Research) effective July 1, 2008, and will be available on a much more expanded basis in the future. Modifications to the protocol and more importantly, to the processing technique will expand patient recruitment. A no-cost extension of the grant from May, 2008, to May, 2009 has been granted.

KEY RESEARCH ACCOMPLISHMENTS

Research objective #1: Establish and validate the micro-array studies, using the animal model. *Deliverable*: A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation**.**

1. Work has continued on preliminary studies relating to this work. These relate to the animal model used, and to previously-employed agents. (See list of publications, below).
2. Collaboration has been continued with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, Kansas. This facility carries out micro-array analysis of both animal and human genomes.
3. It should be noted that the response in all four experimental groups was quite marked, even in the sham group. For example, these are the genes upregulated and downregulated at 30 minutes versus baseline for the four groups. The microarray analysis used 31,099 probes. These data, which are typical of the entire data set, indicates that the sham procedure was a strong stimulant to the gene response. As will be seen below, the pattern of the response to shock and to shock with either from of resuscitation was similar to the response to the sham procedure. There has been a great deal of work published in the trauma literature indicating that shock procedures are, in fact, a combination of trauma and shock. These results tend to confirm that commonly-held belief. However, as noted below, when one goes to analysis of individual genes, it is quite feasible to identify genes which are differentially responsive to sham, shock, and shock with resuscitation. Also refer to the pathway analysis, below.

Experimental group	Genes upregulated	Genes downregulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + arginine at 30 minutes vs baseline	6143	6325

4. The list of signature genes has been defined. The methodology for identifying these genes was as follows. All of the gene responses were uploaded into a web-based system maintained by Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com ; see also below, #8). This system combines data analysis with an extensive genome knowledge base. Genes which showed a strong up-regulation or down-regulation (at least fivefold) to shock, as compares with sham controls, were selected. They were considered as possible signature genes if they showed a difference in their response to shock and to one of the two treatment arms, either fluid resuscitation (shock + LR) or fluid resuscitation plus arginine (shock + arginine). The genes meeting these criteria numbered 390. Selection among these was then carried out by eliminating genes which appeared to be irrelevant to the inflammatory reaction, such as oncogenes. Some genes were selected because they appeared to be related to the known components of the

inflammatory reaction (cytokines).   Several genes were kept on the list because their response in this shock model suggested that they would be good markers of the response to the surgical preparation and to shock (B3GALNT, F3, GRID2, GRM5).   The final list of 20 "signature" genes (below) are to be considered representative of genes which react strongly to the shock preparation, and which appear to be modified by treatment. Further animal work will allow this list to be significantly improved; it has been modified over the past three months, in fact.

Signature Genes

Gene	Protein and function
ALOX5	Arachidonate 5-lipoxygenase (cytoplasmic enzyme)
B3GALNT1	Beta 1,3-N-acetyl/galactosaminyltransferase 2, membrane-bound galactotransferase enzyme
CXCL3	Chemokine (C-X-C motif) ligand 3, chemokine, cytokine
CXCL11	Chemokine (C-X-C motif) ligand 11, chemokine, cytokine
F3	Coagulation factor III, membrane receptor
GRID2	Glutamate receptor, ionotrophic, delta 2 (ion channel)
GRM5	Glutamate membrane receptor
HSPA1B	Heat shock 70kDa protein 1b
HSPBAP	HSPB (heat shock 27kDa) associated protein 1, binds HSPB1
IL1A	Interleukin-1, alpha, cytokine
IL1R2	Interleukin 1 receptor, type II, membrane receptor
MSR1	Macrophage scavenger receptor 1, membrane receptor
NOS2A	Nitric oxide synthase 2A (inducible), cytoplasmic enzyme
NLGN1	Neuroligin 1, membrane receptor (neuronal)
NPY1R	Neuropeptide Y receptor Y1, membrane receptor (G-protein coupled)
NR4A3	Nuclear receptor (subfamily 4, group A, member 3), steroid-thyroid hormone receptor superfamily
PLAUR	Plasminogen activator, urokinase receptor, membrane receptor
RGS1	Regulator of G-protein signaling 1, membrane regulatory protein
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3, membrane glycosaltransferase enzyme
TNF	Tumor necrosis factor (TNF superfamily, member 2), cytokine

5. To facilitate future work in this area, an assay system incorporating RT-PCR is being developed, to allow multiple studies of these signature genes. (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). In this system, 96-well plates will be made up, to incorporate the above genes, each replicated four times. There will be 4 genes used as standards ("housekeeping" genes), which will be genes un-affected by the surgical preparation, shock, or treatment. The response of the signature genes will be referenced to these standard genes.

This system will allow four different samples to be compared on one plate or, alternatively, four replications of a single sample to be determined. It is anticipate that this system will provide validation of the microarray studies, but more importantly, will enable future studies to be done with considerably better accuracy and reproducibility.

6. Data were analyzed using pathway analysis. (Ingenuity Pathways Analysis, Ingenuity Systems, www.ingenuity.com). (Refer to Figures 1-4, below) These analyses are done in terms of the so-called canonical pathways, which are pathways sufficiently well-defined to be considered known and proven. Ingenuity Pathways lists more than 160 pathways. In these experiments, significant responses were seen in around 25 pathways. Figure 1 indicates the seven pathways in which the greatest number of genes were up-regulated or down-regulated in response to shock. These are arranged in order of the pathway responses to shock without resuscitation, at 30 minutes. However, the graphs include all of the groups and time intervals. A number of points should be made.

   a. The response in the sham group was much greater than anticipated. This indicates that the combination of anesthesia and the surgical preparation is itself a powerful stimulant to the organism.
   b. The response to shock, shock plus resuscitation, and shock plus resuscitation plus arginine was different in degree from the response in the sham group, but not in pattern. That is, the pathways showing response to shock also showed response to the surgical preparation itself. This is illustrated in Figure 1.
   c. Within each pathway, there is a distinctive pattern of upregulation and downregulation. Figures 2 through 4 illustrate this, showing a portion of the Hepatic Fibrosis / Hepatic Stellate Cell Activation pathway. In each pathway, the blue color represents down-regulation, and the yellow color represents upregulation. The occasional "mixed" color means that more than one gene contributes to the box on the pathway representing an enzyme, or a receptor complex, but the predominant color represents the predominant response..
   d. There is considerable difference in the gene responses within the pathway, depending on the experimental condition. Figure 2 can be seen the response to shock alone. Figure 3 shows the response to shock with resuscitation using Ringer's lactate. And Figure 4 shows the response to shock with resuscitation with added arginine. In Figure 1, it can be seen that the detailed response of genes in this pathway is different for the three conditions (bars #2, 3, and 4, respectively) The patterns of gene up-regulation and down-regulation change with the treatment.

7. Studies using dietary enrichment with omega-3 fatty acids were completed (funded by another grant) and have been reported at a research workshop and at a national meeting. The relevance of these studies is that these compounds are available in Europe as an intravenous preparation, and they could potentially be used as a component of resuscitation therapy.

■ Sham30 ■ Shock30 ■ Shock+LR30 ■ Shock+arginine30 ■ Sham4h ■ Shock4h ■ Shock+LR4h ■ Shock+arginine

-log(p-value)
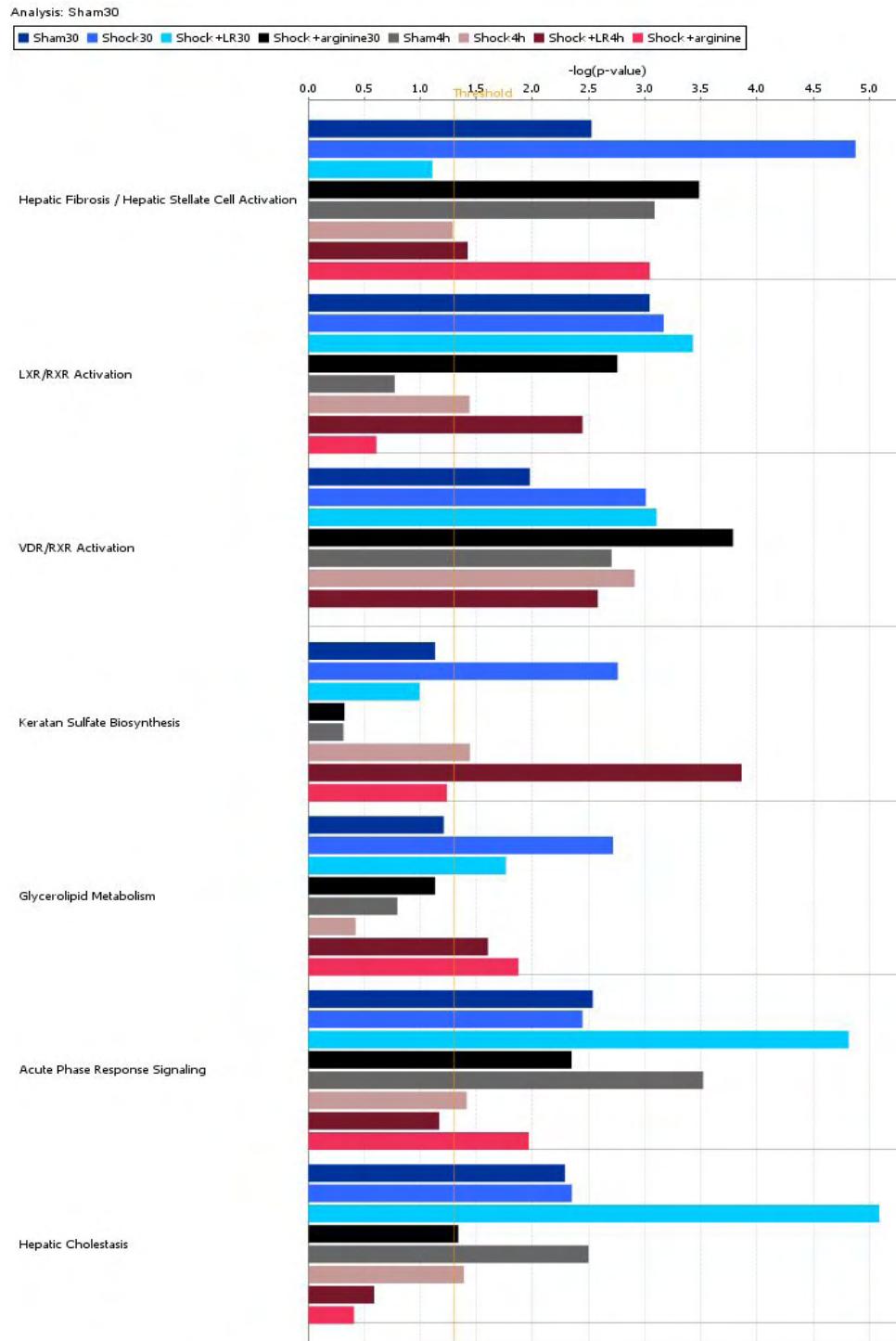
Figure 1: Relative activation of genes in the seven pathways most affected by shock., compared in each group with the baseline values for that group. The first four bar graphs for each pathway represent sham 30 minutes, shock 30 minutes, shock resuscitated with LR at 30 minutes, and shock resuscitated with LR and arginine at 30 minutes. The second four bar graphs represent the same experimental groups at 4 hours. Most of the pathways show an attenuated response at four hours.

Figure 2 A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates upregulation, and the blue color downregulation. This shows the response to shock without resuscitation at 30 minutes, as compared to baseline for that group.

Figure 3. A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates upregulation, and the blue color downregulation. This shows the response to shock with resuscitation using Ringer's lactate at 30 minutes.
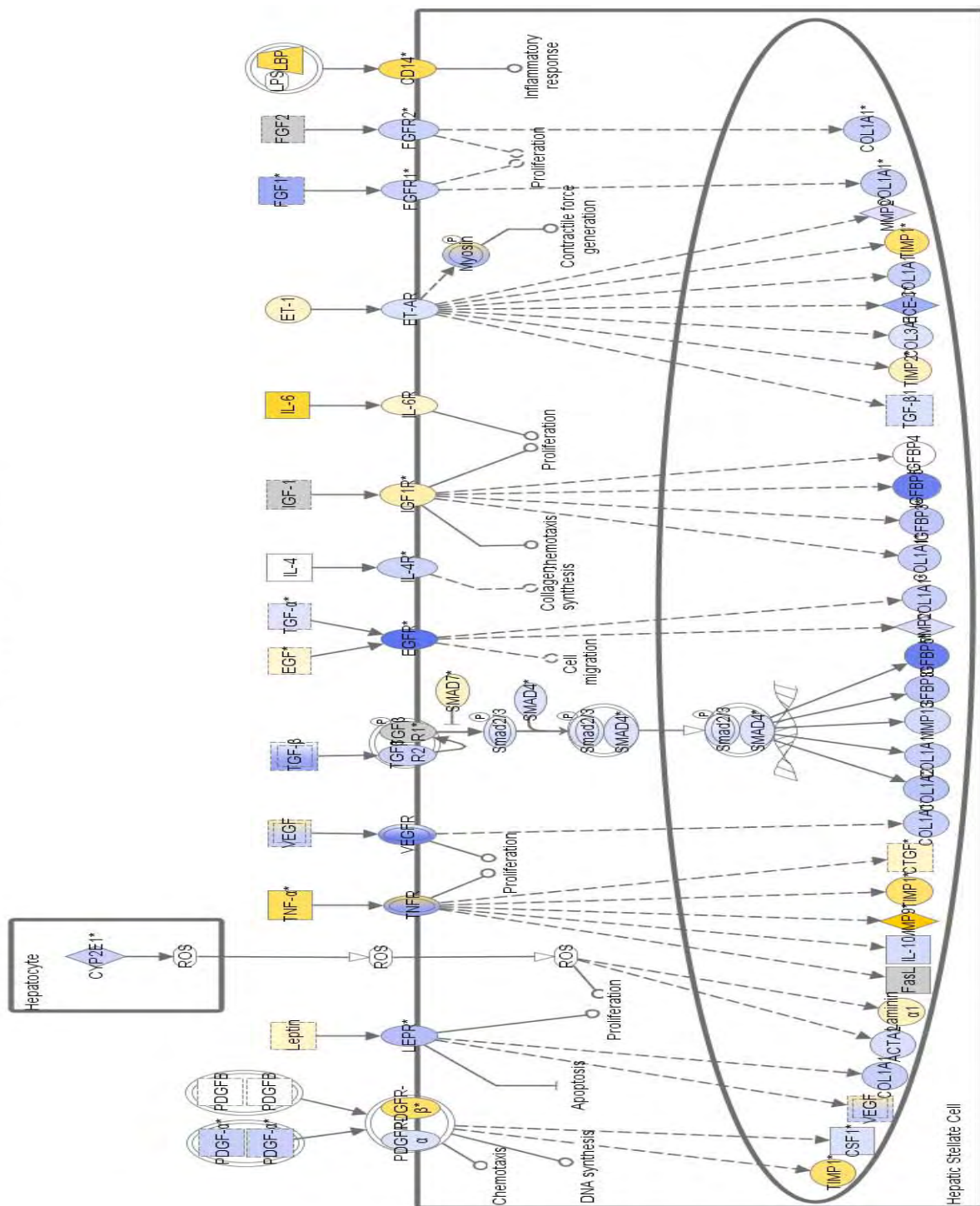
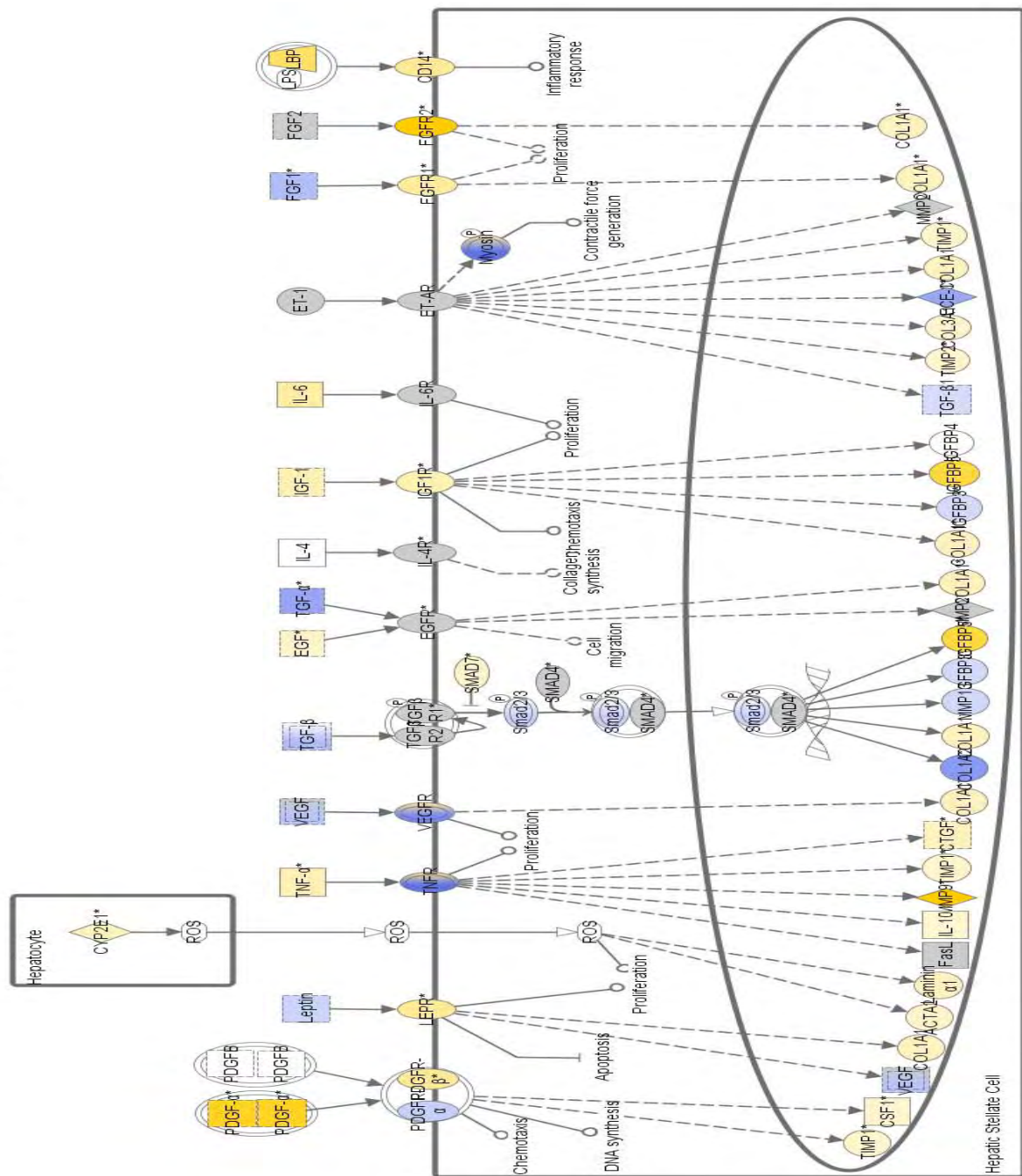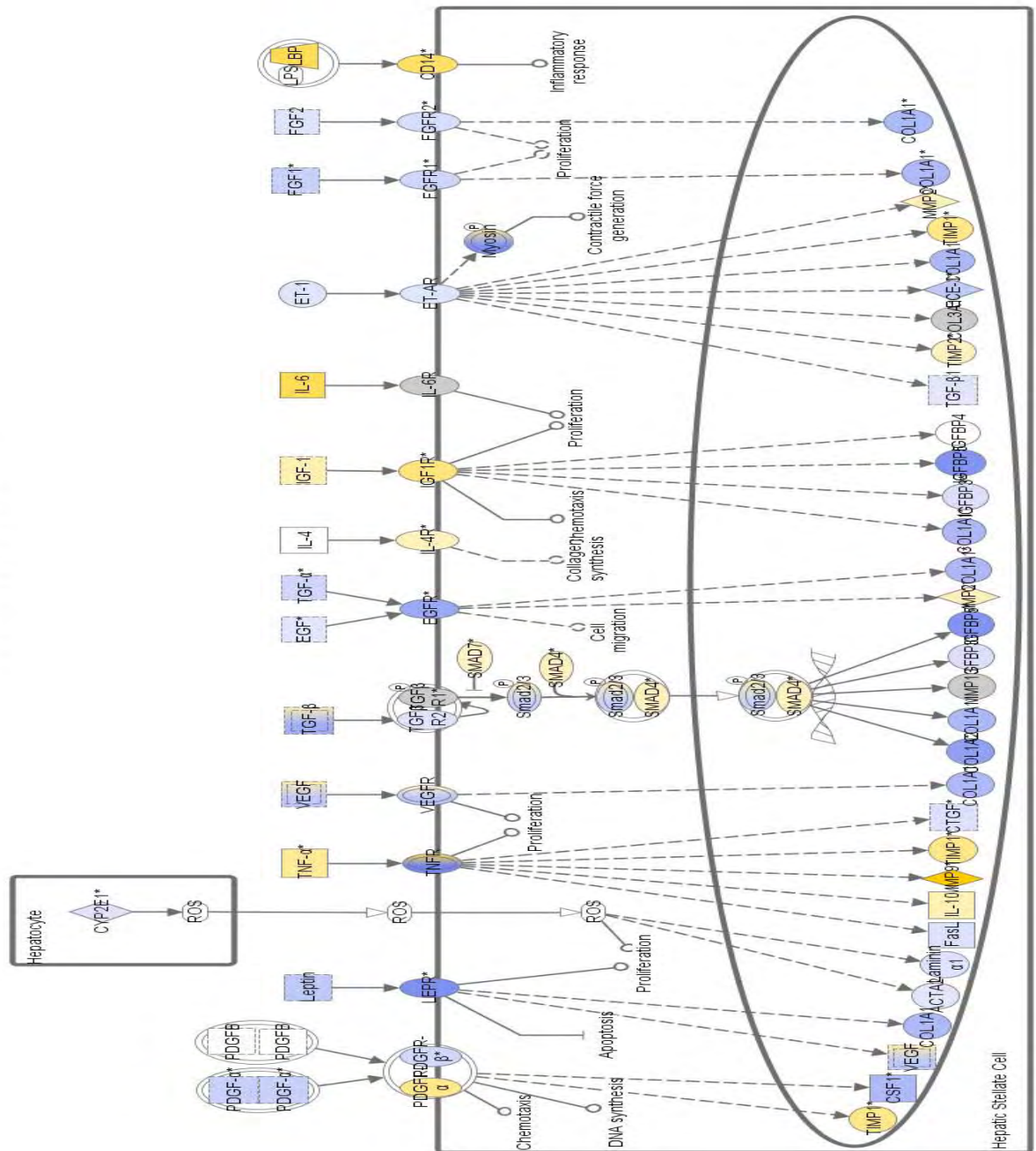Figure 4. A portion of the Hepatic Fibrosis/Stellate Cell Activation pathway, showing the hepatic stellate cell, with genes activated in the extracellular fluid, the cell membrane, and the nucleus. Here, the yellow color indicates upregulation, and the blue color downregulation. This shows the response to shock with resuscitation and arginine, at 30 minutes, as compared to the baseline for the same group.

Research objective #2: Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable*: The effect of the agents upon the level of expression of the signature genes.

1. The choice of the agents has been refined. Alanine-glutamine and glutamine are still being investigated. While alanine-glutamine has major advantages as a drug for administration, glutamine appears, of the two, to have a superior effect on survival in the rat model. It will therefore be used for further studies. Related to this is the issue of approval by the Food and Drug Administration. Glutamine can be used in humans, while alanine-glutamine dipeptide is not approved in the United States. Crocetin is still in early Phase I studies, and will be unavailable for human studies within the timeline of this study. Arginine is a n amino acid which strongly ameliorates the inflammatory reaction, has shown benefit in the rat model. Mevinolin (Lovastatin), which has been used in experimental septic shock by Dr. Qureshi, has been a candidate for this study, but it appears that it will be impractical to administer in the clinical situation because it is not available in an intravenous form. Finally, allopurinol is available in an intravenous form, and will be used in the further studies. This drug was shown to provide survival benefit in the canine shock model 20 years ago, by the PI and others (Allan G, Cambridge D,Van Way CW, Whiting M: The protective action of allopurinol in an experimental model of haemorrhagic shock and reperfusion. British J of Pharmacol, 89:149-155, 1986). This agent has been used to ameliorate the harmful effects of hypoxia in harvested organs to be transplanted (as in the standard UW Solution) and in cold cardioplegia during aortic valve surgery. It appears to act by inhibiting xanthine oxidase, and hence lowering the production of free radicals during reperfusion. Dihydro-epi-andosterone (DHEA) was used in a whole-animal porcine model, in earlier work from our laboratory, but its use was disappointing, and its further use in patients cannot be recommended on the evidence available.

2. Survival studies with each of these agents have been carried out. This was done in the rat model for glutamine and arginine. Previous work by the PI has shown survival benefit for allopurinol in both the canine shock model and the rat model.

3. Glutamine studies are presented in this table.

Survival with or without Glutamine in Resuscitation

	LR	Glutamine
Rat weight (gram)	295.83±3.03	293.00±3.84
Cannulating Time (minutes)	6.50±0.29	6.50±0.23
Bleeding (ml)	9.22±0.41	9.05±0.58
*Survival > 24 hours	4/12	11/12

Shock at MAP = 22±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without glutamine (630mg/kg) over 30 minutes. Data expressed as mean ± SEM. *P< 0.05 (LR *vs* Glutamine).

4. Alanine-glutamine dipeptide (AGD) studies are presented here. It should be noted that AGD is used widely in the clinical situation (in Europe, not in the US), and that it appears to be cleaved to alanine and glutamine once it enters the circulation. It is thus

considered to be therapeutically equivalent to glutamine. However, the present studies show that, while AGD is definitely of benefit, it does not have as pronounced an effect on survival as glutamine.

Survival with or without AGD in Resuscitation

	LR	AGD
Rat weight (gram)	294.61±2.47	297.58±3.48
Cannulating Time (minutes)	6.78±0.27	6.58±0.29
Bleeding (ml)	10.14±0.29	10.93±0.43
***Survival <24 hours**	10/18	4/18
Survival > 24 hours	6/18	9/18

Shock at MAP = 25±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without Alanine-glutamine dipeptide (AGD 936mg/kg, equivalent with glutamine 630mg/kg) over 30 minutes. Data expressed as mean ± SEM. *$P< 0.05$ (LR *vs* AGD).

5. Arginine studies are presented here. In this model, arginine was nearly as effective as glutamine.

Survival with or without Arginine in Resuscitation

	LR	Arginine
Rat weight (gram)	286.70±4.03	294.40±4.81
Cannulating Time (minutes)	6.35±0.13	6.80±0.17
Bleeding (ml)	9.32±0.31	10.06±0.35
***Survival < 24 hours**	11/20	4/20
Survival > 72 hours	6/20	12/20

Shock at MAP = 20±4 mmHg, 90 minutes. Resuscitated with Ringer's Lactate (LR, 21ml/kg) with/without arginine (300mg/kg) over 30 minutes. Data expressed as mean ± SEM. *$P< 0.05$ (LR *vs* Arginine).

6. A cell-culture based model has been developed, using a standard macrophage cell line (RAW 264.7). This model is based on exposing cultured macrophages to ischemia in a sealed chamber, and then re-exposing them to levels of oxygen found in room air (21%) For these experiments, it has been determined that reducing the level of oxygen to 1.5% from the usual 21% appears to produce a significant hypoxic insult. This corresponds to a partial pressure of oxygen of 10 mm Hg. By measuring the effect of therapeutic agents on hypoxia-challenged cell lines, their effects on cytokine production, on caspase 3/7 and caspase 9, and signature genes will provide further evaluation of their possible therapeutic effectiveness. Examination of this model is in the preliminary stages, but several significant observations have been made.

a. First, cells exposed to hypoxia for four hours and then kept in normal oxygen (21%, or about 140 mm Hg partial pressure) for 24 hours and 48 hours show the same growth as control cells. Hypoxic cells grow at a normal rate for 24 hours but their growth slows markedly between 24 and 48 hours, as compared to controls.

b. Second, measuring energy levels within the cells suggests that ATP in the ischemic cells is depressed at the end of the ischemic period. After re-oxygenation, the ischemic cells recover ATP, when compared with cells kept ischemic for the entire experiment. However, because of the differential effects on growth, further studies need to be done to determine the extent of the ATP depression, and the effect of re-oxygenation on ATP recovery. In the whole animal model of shock, ATP is also depressed, and after resuscitation, recovery is slow and incomplete compared with un-shocked controls.

c. Third, stimulating the cells (with lipo-polysaccharide) during the re-oxygenation process produces elevated levels of caspase 3 and 7 compared with both normoxic controls and with hypoxic controls. This is consistent with increased caspase activation and increased apoptosis seen in the whole animal shock model.

d. Studies of cytokine response to LPS are ongoing, and will include TNF-alpha and IL-2 beta. Ischemia alone does not stimulate cytokine production in these cells, but appears to influence the response to LPS..

e. Ischemia produces a differential response to iNOS.

Research objective #3: Assess gene expression in patients treated with current initial resuscitation protocols. *Deliverable*: Coordinated with objective #1, A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.

1. The experimental protocol was approved by the Institutional Review Board of the University of Missouri – Kansas City. It is has taken a considerable amount of time for the pre-approval review by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC).

2. Initial attempts to implement the protocol met with great difficulty enrolling patients, for a number of reasons. A major barrier has been the simple fact that most patients arrive at night or on weekends. However, a commercial blood processing system is being introduced. (LeukoLOCK Total RNA Isolation System, Ambion, Austin TX). This system allows the investigators to extract the white blood cells using a simple filter technique, inactivate the cells using a commercial hypertonic preservation agent (RNAlater, Ambion, Austin, TX), and keep the sample refrigerated for several days without degradation or alteration of the mRNA. The protocol is being modified to incorporate this methodology.

3. Under this protocol, blood samples will be taken from patients arriving in the trauma center who are injured severely enough to exhibit blood loss or signs of

hypovolemia. Blood samples will be collected one and four hours after resuscitation. Tissue samples (liver and muscle) will be taken if the patient is brought to the operating room for immediate operative therapy. These samples will then be processed and submitted for microarray analysis.

Research objective #4: Assess the response of patients to administration of modified resuscitation procedures. *Deliverable*: The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

This objective has not been started, as its initiation has to follow completion earlier objectives. The approval process, has begun, however.   On the basis of work carried out so far, glutamine, arginine, and allopurinol will be the agents selected for human use. All three have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans in the doses contemplated.

REPORTABLE OUTCOMES

PUBLICATIONS AND PRESENTATIONS

1.   Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1-beta, and inducible nitric oxide synthase in hemorrhagic shock. *JPEN J Parenter Enteral Nutr.* 2006 Jul-Aug;30(4):297-301.

2.   Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Helling T, Van Way CW 3rd. Effect of DHEA on the hemodynamic response to resuscitation in a porcine model of hemorrhagic shock. *J Trauma.* 2006 Dec;61(6):1343-9.

3.   Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. *Clinical Nutrition Week of the American Society for Parenteral and Enteral Nutrition*, Dallas. February 12-15, 2006. (Oral presentation). *Clinical Nutrition week,.* 2006, p145. (Abstract of Distinction)

4.   Thomas A, Yang R, Van Way CW III, Quresh N. A Preliminary micro-array study of the response to hemorrhagic shock and to therapy with crocetin and alanine-glutamine dipeptide. *Kansas City Area Life Sciences.* March 30, 2006. *Kansas City Life Sciences.* 2006, p163. (Abstract)

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6.   Yang R, Thomas A, Tan X, Landis M, Qureshi N, Morrison D, Van Way CW III. Hemorrhagic shock in the rat: comparison of femoral and carotid techniques. *Twenty-ninth Annual Conference on shock*. Broomfield, Colorado. June 3-6, 2006. *Shock*. 25:S97,2006. (Abstract)

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8.   Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. *JPEN J Parenter Enteral Nutr.* 31:94-100,2007.

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13. Qureshi N, Reis J, Shen J, Monaco JJ, Papasian CJ, Qureshi AA, Vogel SN, and Morrison DC. Cutting Edge: Differential regulation of LPS-induced TNF and NO by proteasomes. (*Shock*, reviewed, acceptable after changes) 2008.

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17. Van Way CW, Yang R, Harris WS, Almenoff M, Xu D, Vernon K, Thomas AM, Qureshi N, Morrison DC. Pre-feeding with omega 3 fatty acids suppresses inflammation from hemorrhagic shock. American Society for Parenteral and Enteral Nutrition, Feb 10-13, 2008. (Chicago, IL) (Presented by C. Van Way)

18. Van Way CW, Harris WS, Yang R, Almenoff M, Xu D, Vernon K, Thomas AM, Qureshi N, Morrison DC. Fish oil feeding modulates inflammation in a rat model of hemorrhagic shock. ISSFAL 2008 (8th meeting of the International Society for the Study of Fatty Acids and Lipids) May 17-22, 2008. (Kansas City, MO) (Presented by C. Van Way)

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3. Yang R, Yan X, Thomas AM, Steppacher R, Qureshi N, Morrison DC, Van Way CW 3rd. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. *JPEN J Parenter Enteral Nutr.* 2007 (Jan-Feb) 31(1):32-6.

4. Yang R, Martin L, Woodall C, Thomas AM, Qureshi N, Morrison DC, Van Way CW III. Administration of glutamine following hemorrhagic shock restores cellular energy, reduces cell apoptosis and damage, and increases survival. *JPEN J Parenter Enteral Nutr.* 31:94-100,2007.

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CONCLUSION

This study has rested on two basic hypotheses. The first is that agents given during and (for a short period of time) after resuscitation can favorably influence the response to hemorrhagic shock and injury. The second is that studies of gene response studies, specifically microarray and RT-PCR studies, could be used to assess the response of patients being treated for injuries to such therapeutic agents.

To date, both hypotheses have been shown to be true in the animal model. Work continues to determine optimum therapeutic agents to be used during and after resuscitation. In addition, development has been well begun on a multiple-gene RT-PCR assay incorporating a group of signature genes which have been identified as uniquely responsive to shock and therapy.   Development of this assay will greatly simplify future work in this area, both in our laboratories and in those of other investigators.

Studies are ongoing to validate these hypotheses in patients.   During the next year, which will be a no-further-cost extension of the grant, we will continue this work. The gene studies will be subjected to validation, and the use of three specific therapeutic agents will be subjected to preliminary clinical trial.

While this work has not gone as rapidly as hoped, and while no research in the area of shock proceeds without setbacks, we anticipate considerable progress in this research over the next year.

Quarterly Report

1. Award No.   W81XWH-06-1-0530

2. Report Date: 5/19/2008

3. Reporting period: 2/20/2008-5/17/2008

4. Principal Investigator: Dr. Charles VanWay, III

5. Telephone No.:   816.404.5364

6. Award Organization: The Curators of the University of Missouri

7. Project Title: Early Treatment in Shock

8. Current staff, role and percent effort of each on project.

STAFF MEMBER	Role	% EFFORT
Charles Van Way, MD	PI	25
Nilo Qureshi, PhD	Research Director	15
Kim Dyer, RN	Research Nurse	50
Kathy Vernon, BS	Research Associate	100

9. Contract expenditures to date (as applicable):

COST ELEMENTS	THIS QUARTER	CUMULATIVE
Personnel	21,311.07	204,253.71
Fringe Benefits	6,156.01	58,450.63
Supplies	4,550.76	39,179.14
Equipment	0	13,685.55
Travel	0	0
Other Direct Costs	33,251.60	274,799.23
Subtotal	65,269.44	590,368.26
Indirect Costs	30,634.08	271,040.87
Fee	0	0
Total	95,903.52	861,409.13

10. Comments on administrative and logistical matters.

This report represents a correction to the cumulative amount on Fringe Benefits. It was overstated on the Third Quarter report.

11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible. **Please see annual report, attached.**

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Item 12: Goals for the next quarter

Research objective #1: Establish and validate the micro-array studies, using the animal model. *Deliverable*: A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation**.**
1. The panel of "signature genes" will be additionally validated in the rat model using assays of tissue, specifically liver and skeletal muscle, to determine their usefulness in these tissues.
2. As noted in some detail in the annual report, the panel of 20 "signature genes" is currently being mapped to a custom 96-well plate, using a commercially . (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). The custom plates will incorporate the above genes, each replicated four times. There will be 4 genes used as standards ("housekeeping" genes). These will be genes un-affected by the surgical preparation, shock, or treatment.   The response of the signature genes will be referenced to these standard genes.   This system will allow four different samples to be compared on one plate or, alternatively, four replications of a single sample to be determined. It is anticipate that this system will provide validation of the microarray studies, but more importantly, will enable future studies to be done with considerably better accuracy and reproducibility.

Research objective #2: Define the effect on gene expression of the agents which will be used clinically, using the animal model. *Deliverable*: The effect of the agents upon the level of expression of the signature genes.
2. Gene array analysis for allopurinol, glutamine, and the combination of glutamine, allopurinol, and arginine will be carried out, using the hemorrhagic shock model in the rat. Full micro-array analysis will be supplemented and validated by the analysis of signature genes with RT-PCR, using the TaqMan technology.
3. The TaqMan assay will be used to validate the arginine assay, above.
4. Work will be continued on a cell-culture based model using a standard macrophage cell line (RAW 264.7), described in the annual report.

Research objective #3: Assess gene expression in patients treated with current initial resuscitation protocols. *Deliverable*: Coordinated with objective #1, A panel of genes that are reproducibly altered in white blood cells and in liver and muscle by shock and resuscitation.
1. The protocol for this portion of the study has been pre-approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC), has received final

approval from the Institutional Review Board (IRB) of the University of Missouri – Kansas City (UMKC), and has received final approval from the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC). As noted below, modifications are being made.

2. Blood samples are to be taken from patients arriving in the trauma center who exhibit blood loss or signs of hypovolemia. Under this protocol, blood samples will be taken after resuscitation and at 24 hours and 7 days, from patients arriving in the trauma center who exhibit blood loss or signs of hypovolemia. These samples will then be processed and submitted for microarray analysis. To date, two patients have been entered in this protocol, which is anticipated to enroll 5 to 10 patients. Enrollment has been unexpectedly difficult, due to the requirement that samples must be processed immediately to preserve the RNA, and personnel departures in the laboratory have limited the availability of such processing.

3. Sample processing methods are being simplified. A major barrier to patient enrollment has been the simple fact that most patients arrive at night or on weekends. However, a newly-available commercial blood processing system will permit bypassing this problem. (LeukoLOCK Total RNA Isolation System, Ambion, Austin TX). This system allows the investigators to extract the white blood cells using a simple filter technique, inactivate the cells using a commercial hypertonic preservation agent (RNAlater, Ambion, Austin, TX), and keep the sample refrigerated for several days without degradation or alteration of the mRNA. The protocol is being modified to incorporate this methodology.

Research objective #4: Assess the response of patients to administration of modified resuscitation procedures. *Deliverable*: The effect of the experimental agents upon signature genes, cellular response, and cellular damage in patients.

1. This objective has not been started, as its initiation has to follow completion earlier objectives. The approval process, has begun, however.   On the basis of work carried out so far, glutamine, arginine, and allopurinol will be the agents selected for human use. All three have shown improvement in survival in whole-animal models of shock, all can be given intravenously, and all are safe when given to humans in the doses contemplated. During the coming quarter, the pharmacy at Truman Medical Center will be used to prepare these agents for administration to patients.

c. **FORMAT REQUIREMENTS FOR ANNUAL AND FINAL REPORTS (MAY 1996) USAMRAA**

1) Annual reports must provide a complete summary of the research accomplishments to date with respect to the **approved** Statement of Work.   Journal articles **can be** substituted for detailed descriptions of specific aspects of the research, but the original articles **must** be attached to the report as an appendix and appropriately referenced in the text. The importance of the report to decisions relating to continued support of the research cannot be over-emphasized. A report shall be submitted within

30 calendar days of the anniversary date of the award (a final report will be submitted upon completion of the research (last year of the award).

2) A final report summarizing the entire research effort, citing data in the annual reports and appended publications shall be submitted at the end of the award performance period. The final report will provide a complete reporting of the research findings. Journal publications **can be** substituted for detailed descriptions of specific aspects of the research, but an original copy of each publication **must** be attached as an appendix and appropriately referenced in the text. All final reports must include a bibliography of all publications and meeting abstracts and a list of personnel (not salaries) receiving pay from the research effort.

3) Although there is no page limitation for either the annual or final report, each report shall be of sufficient length to provide a thorough description of the accomplishments with respect to the approved Statement of Work. Submission of an original and two copies of the report are required. Reports shall be forwarded to:

Commander, U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, MD 21702-5012

4) All reports **shall** have the following elements in this order: front cover, Standard Form (SF 298), table of contents, introduction, body, key research accomplishments, reportable outcomes, conclusions, references, and appendices. Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES BUT DO INCLUDE THE APPENDICES IN THE PAGE COUNT IN BLOCK 15 ON THE SF 298.** Mark all pages of the report that contain proprietary or unpublished data that should be protected. **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS.** Indicate in your letter accompanying the report that the report contains proprietary or unpublished data, and that the distribution statement should indicate the limitations of the report.

FRONT COVER: Sample front cover provided at http://mrmc-www.army.mil/rrpindex.asp. The Accession Document (AD) Number should remain blank.

STANDARD FORM 298: Sample SF 298 provided at http://mrmc-www.army.mil. The abstract in Block 13 **must** state the purpose, scope, major findings and be an **up-to-date** report of the progress in terms of results and significance. Subject terms are keywords that may have previously assigned to the proposal abstract or are keywords that may be significant to the research. The number of pages shall include all pages that have printed data (including the front cover, SF 298, table of contents, and all appendices). Please count pages carefully to ensure legibility and that there are no missing pages as this delays processing of reports. **Page numbers should be typed: please do not hand number pages.**

TABLE OF CONTENTS: Sample table of contents provided at http://www.army.mil/rrpindex.asp.

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the **approved** Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Appended publications and/or presentations **may** be substituted for detailed descriptions but **must** be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although the Grants Officer must approve changes to the original Statement of Work. This approval must be obtained prior to initiating any change to the original Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

> manuscripts, abstracts, presentations; patents and licenses applied for and/or issued; degrees obtained that are supported by this award; development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc.; funding applied for based on work supported by this award; employment or research opportunities applied for and/or received based on experience/training supported by this award.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.